Phenotypic and genotypic characterization of *Salmonella* spp. isolated from foods and clinical samples in Brazil

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ABSTRACT

Increasing antimicrobial resistance in *Salmonella* species has been a serious problem for public health worldwide. This study examines *Salmonella* spp. recovered from foods and clinical samples on serotype, antimicrobial resistance and PFGE genotypes. It identified 91 salmonellae, belonging to 31 different serotypes, from 36 isolates from food and 55 clinical samples. *Salmonella* Infantis (16.5%) and *Salmonella* Enteritidis (13.7%) are the most common among food isolates, whereas *Salmonella* Enteritidis (29.0%) and *Salmonella* Typhimurium (16.0%) mainly causes human salmonellosis. Antimicrobial susceptibility data showed that 63.0% of the isolates were fully susceptible to 12 antibiotics tested. Nalidixic acid showed high resistance rates, 32.7% and 25.0% of the clinical isolates and food, respectively. Three main PFGE types: A (*Salmonella* Enteritidis), B (*Salmonella* Infantis) and C (*Salmonella* Schwarzengrund) comprised isolates recovered from foods and clinical samples. Our results indicate that the clonal groups were both causing diseases and food contamination, emphasizing the need to maintain a system of surveillance for foodborne disease.

Key words: *Salmonella*, *Salmonella* Infantis, *Salmonella* Enteritidis, antimicrobial resistance.

INTRODUCTION

Salmonella is a leading cause of food-borne disease being an important health problem in industrialized and developing countries (Chu et al. 2009, Abbassi-Ghozzi et al. 2012, Yang et al. 2013). In recent years, serotypes of *Salmonella* isolated from foods have shown an increase in antimicrobial resistance, particularly those of animal origin. Moreover, an increased incidence of human salmonellosis due to multidrug-resistant strains have been reported (Lu et al. 2011, Wang et al. 2013).

There has been increasing concern over the past 30 years about the worldwide emergence of multidrug-resistant phenotypes among *Salmonella*

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serovars, in particular *Salmonella* Typhimurium and, more recently, *Salmonella* Newport as well as several other serovars. The levels and extent of resistance vary in different regions and are influenced by antimicrobial use in humans and animals as well as geographical differences in the epidemiology of *Salmonella* (Weinberger and Keller 2005, Zhao et al. 2008).

Most individuals with gastroenteritis present no serious clinical conditions and do not need hospitalization, so it is difficult to isolate the causative agents and identify the sources of contamination, thus underestimating the number of cases of salmonellosis worldwide. In Brazil, during the period of 2001 to 2006, a total of 3,749 cases of typhoid fever were reported with 30 deaths, the highest number recorded in the North and Northeast regions, especially in the states of Acre (741 cases), and Bahia (671 cases) (Brazilian Ministry of Health 2009, Souza et al. 2010).

However, sporadic studies and limited information on *Salmonella* food contamination and microbiological investigation in foodborne infections have not clarified uncertainties regard of the salmonellosis epidemiology in Brazil (Campioni et al. 2012). Therefore, the current study evaluates the prevalence of antimicrobial susceptibility and genotypes of *Salmonella* spp. isolated from food and human in the state of Bahia (Northeast region) from 1996 to 2012.

MATERIALS AND METHODS

SAMPLES

This study analyzed 91 isolates of *Salmonella* spp. from various cities in the State of Bahia Brazil, 36 isolated from food (meat, fish, chicken and pork, cheeses, salads, rice, ready-to-eat foods and desserts). Food Isolates came from the Laboratory for Food Microbiology Research, Federal University of Bahia and the Central Public Health Laboratory of Bahia (LACEN / BA). Merchants and

local health surveillance officers sent food samples to the laboratories with suspected food infection outbreaks, isolating them for identification of *Salmonella*. Isolated from clinical specimens (55 isolates) were isolated from human feces (n = 43), cerebrospinal fluid - CSF (n = 10), and blood (n = 2) of patients treated at four hospitals in the city of Salvador, Bahia. Invasive CSF samples were obtained from patients with meningitis and blood and stool samples were obtained from patients with gastroenteritis suspected. The information provided by hospitals or food laboratories were referring to the year of isolation, source (feces, blood, CSF or food) and city of origin.

SEROLOGICAL IDENTIFICATION

The isolates were serotyped by the slide agglutination method using antisera prepared at the National Reference Laboratory for Enterobacteria of the Oswaldo Cruz Foundation in Rio de Janeiro (Fundação Oswaldo Cruz - FIOCRUZ-RJ).

ANTIMICROBIAL SUSCEPTIBILITY

Antimicrobial susceptibility was assessed to ampicillin 10 µg (AMP), cephalothin 30 µg (CFL), cefotaxime 30 µg (CTX), gentamicin 10 µg (GEN), tetracycline 30 µg (TET), chloramphenicol 30 µg (CLO), trimethoprim/sulfamethoxazole 1.25/23.75 µg (TMS), ciprofloxacin 5 µg (CIP), amoxicillin/clavulanate 20/10 µg (AMC), nalidixic acid 30 µg (NLX), levofloxacin 5 µg (LEV) and ceftriaxone 30 µg (CRO). The agar diffusion method according to Clinical and Laboratory Standards Institute guidelines (CLSI 2016). The study adopted strain *Escherichia coli* ATCC® 25922 as reference for quality control.

PFGE TYPING AND ANALYSIS

Chromosomal digests generated by *Xba*I were prepared and analysed as described previously (CDC 2010). Fragments were separated in 1.0%

agarose gels using using CHEF-DRII apparatus (Bio-Rad Laboratories, Hercules, CA) with pulse times of 2.2 to 63.8s for 20h at 14°C and 6 V/cm in TBE buffer. Restriction profiles were analysed using GelCompar II (Applied Maths, St. Martens, Belgium). To develop the dendrogram, the study used an unweighted pair-group with arithmetic mean (UPGMA) method and the Dice similarity coefficient, with a position tolerance coefficient of 1.5%. Isolates with ≥ 90.0% relatedness were considered a cluster.

RESULTS

Out of the 91 samples investigated, it was possible to identify 20 serotypes (Table I) from a set of isolates from food, of which 16.5% (6 of the 36) corresponded to serotype *Salmonella* Infantis (Figure 1).

Among isolates obtained from clinical samples, this study identified 18 different serotypes. *Salmonella* Enteritidis was predominant with 29.1% (Figure 1). Serotype diversity was lower among clinical isolates than in foods samples (Table II).

In regards to antimicrobial resistance, 63.0% (57/91) of the isolates were sensitive to all antibiotics tested and 37.0% (34/91) resistant to at least one of the 12 antibiotics tested (Table III). High resistance rates were seen for nalidixic acid among the isolates, 32.7% and 25.0% of the clinical isolates and food, respectively. In addition, isolates from clinical samples were more resistant than isolates from food: resistance to tetracycline (25.5% vs. 8.3%), ampicillin (21.8% vs. 5.6%) and TMS (14.5% vs. 8.3%). However, amoxicillin/ clavulanate, cephalothin, chloramphenicol, ceftriaxone, cefotaxime and gentamicin showed

TABLE I
Distribution of *Salmonella* spp. serotypes from food isolates (N=36).

Serotypes	Food Source	N° of Isolates	Total	%
Salmonella Infantis	Salami	1	6	16.5
	Cooked rice	1		
	Pork	1		
	Cooked chicken	1		
	Raw chicken	1		
	Uncooked seasoned pork	1		
Salmonella Enteritidis	Peanut pave	1	5	13.7
	Chocolate bonbon	1		
	Chocolate pave	1		
	Homemade cake	1		
	Strawberry syrup	1		
Salmonella Senftenberg	Uncooked pork pepperoni	1	3	8.2
	Charru Mussel	1		
	Raw chicken	1		
Salmonella Mbandaka	Raw fish	1	2	5.6
	Uncooked chicken file	1		
Salmonella Muenchen	Fresh sausage	1	2	5.6
	Uncooked seasoned chicken	1		
Salmonella Newport	Cheese curd	2	2	5.6

TABLE I (continuation)

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Salmonella Schwarzengrund	Raw salad	1	2	5.6
	Raw chicken	1		
Salmonella Typhimurium	Raw salad	1	2	5.6
	Raw beef	1		
Salmonella Agona	Raw chicken	1	1	2.8
Salmonella Cubana	Raw chicken	1	1	2.8
Salmonella enterica subsp. *enterica* (O:6,7)	Raw salad	1	1	2.8
Salmonella enterica subsp. *enterica* (O:6,7:e,h:-)	Cheese curd	1	1	2.8
Salmonella enterica subsp. *enterica* (O:6,7:r:-)	Cheese curd	1	1	2.8
Salmonella enterica subsp. *enterica* (O:6,8)	Uncooked ground beef	1	1	2.8
Salmonella enterica subsp. *enterica* (O:11)	Cooked salad	1	1	2.8
Salmonella Heidelbeg	Cheese curd	1	1	2.8
Salmonella Kentucky	Raw salad	1	1	2.8
Salmonella Livingstone	Smoked meat	1	1	2.8
Salmonella Muenster	Halfbeak fish	1	1	2.8
Salmonella Orion	Sausage	1	1	2.8
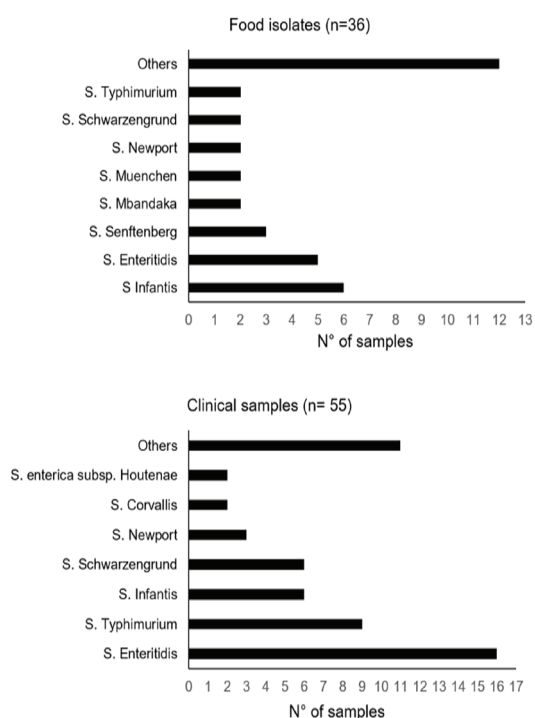
Figure 1 - Distribution of *Salmonella* serotypes in food and clinical samples.

low resistance rates. All isolates were susceptible to ciprofloxacin and levofloxacin.

Among resistant isolates, this study identified 15 patterns among resistant isolates, four of which were found only in isolates from foods (GEN, NLX/TET, NLX/TMS and AMP/TET/TMS). Nine patterns were identified only in clinical isolates (NLX, TET, NLX/TET, TET/TMS, AMP/NLX/TET, AMP/TET/TMS, AMP/NLX/TMS, AMP/AMC/CLO/TET and AMP/GEN/NLX/TET), and two patterns were common to both groups (NLX and AMP/CFL/CRO/CTX/TET/TMS/NLX). The most frequently observed pattern was NLX identified among *Salmonella* Enteritidis isolates from food (16.0%) and clinical samples (11.0%). Overall, antibiotic resistance between clinical and food isolates showed that clinical isolates are more resistant to antibiotics (Figure 2). The *Salmonella* Schwarzengrund showed multidrug resistance with resistance to phenotype AMP/CFL/CRO/CTX/

TABLE II
Distribution of serotypes of *Salmonella* spp. isolated from clinical samples (N= 55).

Serotypes	Sample Source	Nº of Isolates	Total	%
Salmonella Enteritidis	Stool	11	16	29.1
	CSF	5		
Salmonella Typhimurium	Stool	6	9	16.4
	CSF	3		
Salmonella Infantis	Stool	6	6	10.9
Salmonella Schwarzengrund	Stool	6	6	10.9
Salmonella Newport	Stool	3	3	5.5
Salmonella Corvallis	Stool	2	2	3.7
Salmonella enterica subsp. *Houtenae*	Blood	1	2	3.7
	CSF	1		
Salmonella Agona	Stool	1	1	1.8
Salmonella Anatum	Blood	1	1	1.8
Salmonella Cerro	Stool	1	1	1.8
Salmonella Derby	Stool	1	1	1.8
Salmonella enterica subsp. *enterica* (O:6,7:e,h:-)	Stool	1	1	1.8
Salmonella enterica subsp. *enterica* (O:4,5:-:1,6)	Stool	1	1	1.8
Salmonella Indiana	Stool	1	1	1.8
Salmonella Mbandaka	Stool	1	1	1.8
Salmonella Montevideo	Stool	1	1	1.8
Salmonella Panama	Stool	1	1	1.8
Salmonella Worthington	CSF	1	1	1.8

CSF (Cerebrospinal Fluid)

TET/TMS/NLX found in both the food (3.0%) and clinical isolates (9.0%).

The study identified 47 PFGE types, including 11 clonal groups, ranging from 2 to 20 isolates. The isolates were grouped in two major clusters designated as PFGE A (20 isolates) and PFGE B (11 isolates). In the PFGE A cluster, 19 isolates of *Salmonella* Enteritidis and one *Salmonella* Infantis exhibited similarity above 90.0% (Figure 3).

The PFGE A cluster included isolates from food (4), feces (11) and CSF (5) identified from 1996 to 2011 in different localities of the Bahia State. The PFGE B cluster contained 11 isolates of *Salmonella* Infantis (food [7] and feces [4]) (Figure 4b). The PFGE C cluster contained seven isolates of *Salmonella* Schwarzengrund (food [1] and feces [6]) (Figure 4c). In total, 85.7% of food samples were of animal origin and identified over a three-year period (2009-2011).

DISCUSSION

Salmonella is an environmentally persistent pathogen that that survives and multiplies in diverse environments, and is one of the most common bacteria in food-borne diseases in humans. The CDC estimated that, each year, there are approximately one million cases of salmonellosis caused by non-typhoidal *Salmonella* (CDC 2011). In Brazil, salmonellosis has been substantially underestimated, thus, this study brings important

TABLE III
Antimicrobial resistance profiles for *Salmonella* isolates from food and clinical samples.

Resistance profile	Nº of strain isolated from foods (n = 12/36)	Nº of strain isolated from clinical samples (n = 22/55)
GEN	2	
NLX	6	6
NLX*		1
TET		1
NLX/TET	1	
NLX*/TET		1
NLX/TMS	1	
TET/TMS		1
AMP/NLX/TET		1
AMP/TET/TMS	1	
AMP*/NLX/TMS		1
AMP*/TET/TMS		1
AMP/AMC*/CLO/TET		1
AMP/GEN/NLX/TET		3
AMP/CFL/CRO/CTX/TET/TMS/NLX*	1	5

GEN (gentamicin); NLX (nalidixic acid); TET (tetracycline); TMS (trimethoprim/sulfamethoxazole); AMP (ampicillin); AMC (amoxicillin/clavulanate); CLO (chloramphenicol); CFL (cephalothin); CRO (ceftriaxone); CTX (cefotaxime); *Intermediate sensitivity.

Figure 2 - Resistance of *Salmonella* serotypes in food and clinical samples to a variety of antimicrobials. X-axis represents the number of antimicrobials to which strains are resistant. Isolates from human and food were grouped according to the number of antimicrobial agents they were resistant.

contribution to public health in Brazil and all over the world.

The most frequently observed serotypes in food samples are similar to those reported in other regions of Brazil and around the world (Murakami et al. 2007, EFSA 2011, Pulido-Landinez et al. 2013). Of 16 serotypes identified among food isolates, *Salmonella* Infantis was the most frequent serovar (16.0%), followed by *Salmonella* Enteritidis (14.0%). In r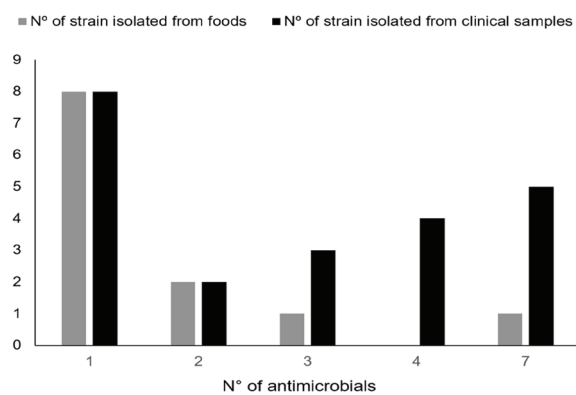egards to clinical samples, the predominant was *Salmonella* Enteritidis (29.0%), followed by *Salmonella* Typhimurium (16.0%), *Salmonella* Infantis (11.0%) and *Salmonella* Schwarzengrund (11.0%).

Derived food products such as poultry meat and eggs are the main forms of infection by *Salmonella* spp. in humans (Gantois et al. 2009). A large number of serovars of *Salmonella* are pathogenic for humans, so the ability to genus *Salmonella* to cause a disease depends on several virulence determinant factors. Therefore, there is a less diverse serotypes among clinical isolates than food samples. Higher frequencies of *Salmonella* Enteritidis and *Salmonella* Typhimurium were also found in other studies in Brazil and elsewhere, and they pose a threat to human health (Silva and Duarte 2002, Mürmann et al. 2009).

Salmonella Enteritidis was the most common serotype in the country. In 1993, *Salmonella* Enteritidis accounted for approximately 10.0% of the *Salmonella* serotypes isolated from human samples, and from then on it became the dominant serotype in both human and non-human samples (Taunay et al. 1996). In 1994 and 1995, it accounted for 43% and 65% of *Salmonella* Enteritidis isolated from humans, respectively, in Sao Paulo
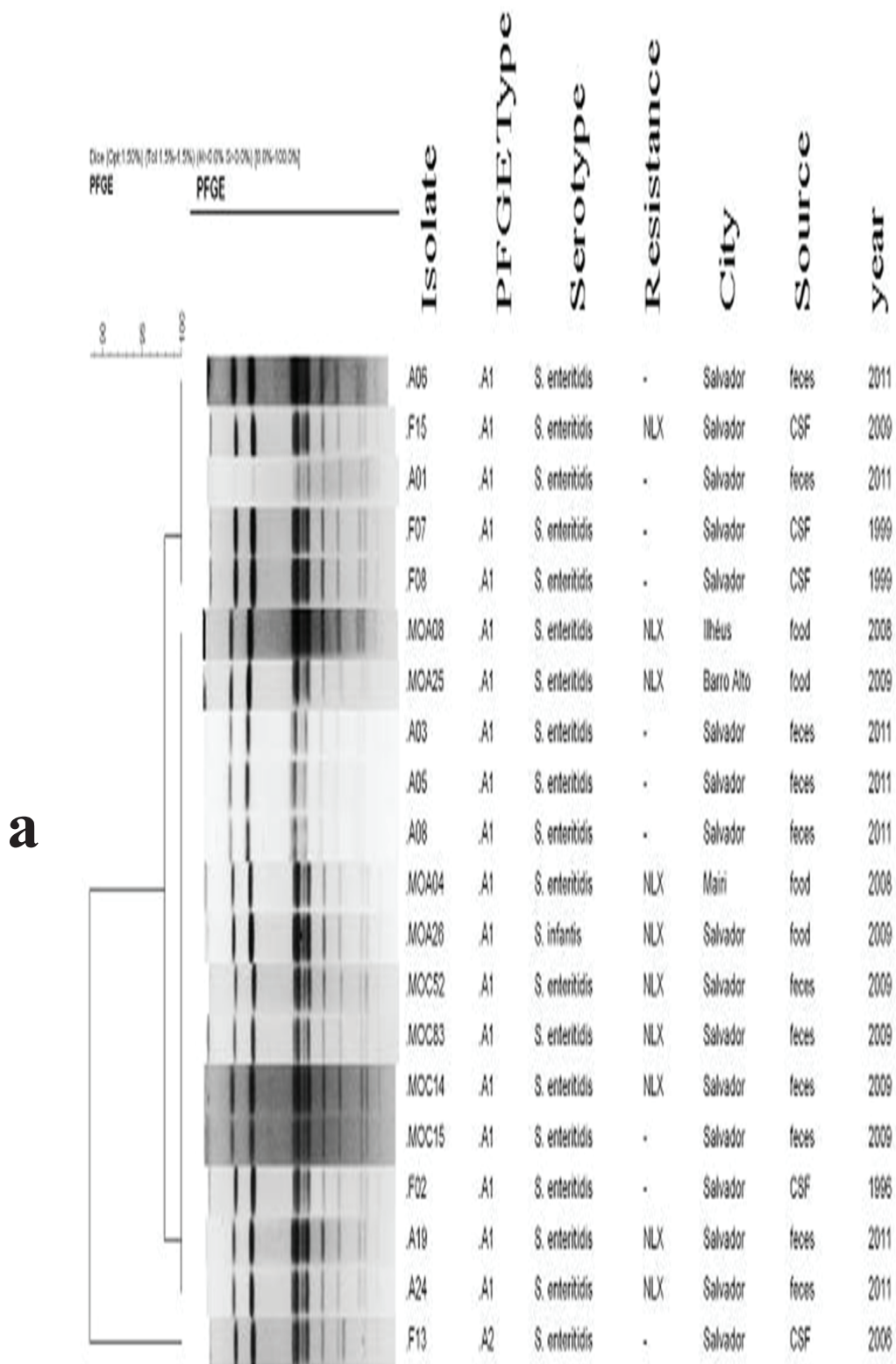
Isolate	PFGE Type	Serotype	Resistance	City	Source	year
A06	A1	S. enteritidis	-	Salvador	feces	2011
F15	A1	S. enteritidis	NLX	Salvador	CSF	2009
A01	A1	S. enteritidis	-	Salvador	feces	2011
F07	A1	S. enteritidis	-	Salvador	CSF	1999
F08	A1	S. enteritidis	-	Salvador	CSF	1999
MOA08	A1	S. enteritidis	NLX	Ilhéus	food	2008
MOA25	A1	S. enteritidis	NLX	Barro Alto	food	2009
A03	A1	S. enteritidis	-	Salvador	feces	2011
A05	A1	S. enteritidis	-	Salvador	feces	2011
A08	A1	S. enteritidis	-	Salvador	feces	2011
MOA04	A1	S. enteritidis	NLX	Mairi	food	2008
MOA26	A1	S. infantis	NLX	Salvador	food	2009
MOC52	A1	S. enteritidis	NLX	Salvador	feces	2009
MOC83	A1	S. enteritidis	NLX	Salvador	feces	2009
MOC14	A1	S. enteritidis	NLX	Salvador	feces	2009
MOC15	A1	S. enteritidis	-	Salvador	feces	2009
F02	A1	S. enteritidis	-	Salvador	CSF	1996
A18	A1	S. enteritidis	NLX	Salvador	feces	2011
A24	A1	S. enteritidis	NLX	Salvador	feces	2011
F13	A2	S. enteritidis	-	Salvador	CSF	2006

Figure 3 - Dendrogram representing genetic relationships among *Salmonella* Enteritidis **(a)** strains based on PFGE. The data were sorted by the UPGMA method. NLX (nalidixic acid).
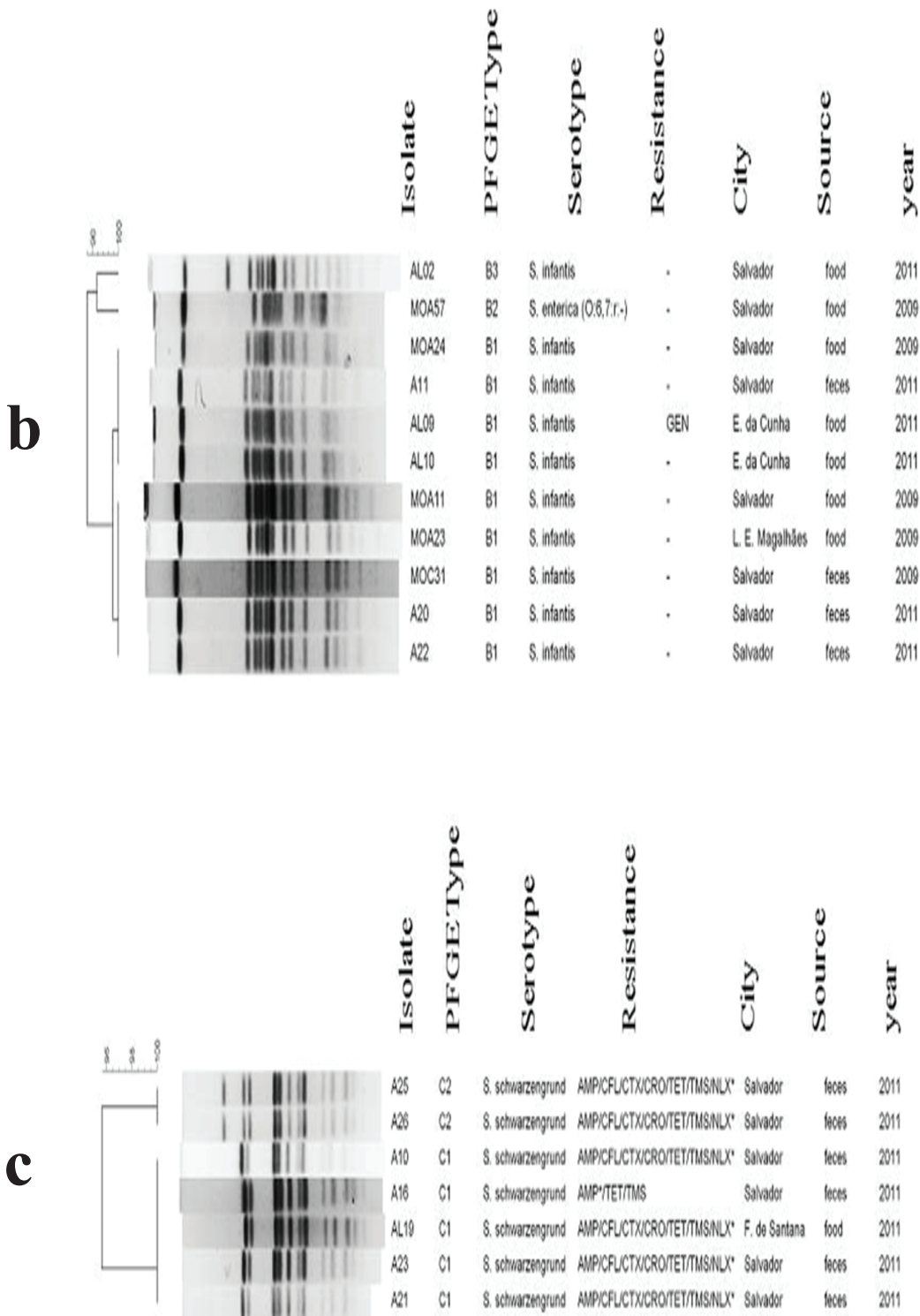
Figure 4 - Dendrogram representing genetic relationships among *Salmonella* Infantis **(b)** and *Salmonella* Schwarzengrund **(c)** strains based on PFGE. The data were sorted by the UPGMA method. GEN (gentamicin); NLX (nalidixic acid); TET (tetracycline); TMS (trimethoprim/sulfamethoxazole); AMP (ampicillin); CFL (cephalothin); CRO (ceftriaxone); CTX (cefotaxime).

(Taunay et al. 1996). Modifications in serotype distribution reflect changes in animal production and the spreading of new serotypes due to world trade. Of a particular concern is the emergence of resistance to multiple antimicrobials. Resistance to quinolones, such as nalidixic acid, has become a significant problem for public health and has been increasing in recent years, mainly due to the liberal use of quinolone drugs in aquaculture and industrial animal production (Oliveira et al. 2005). In contrast, antibiotics such as aminoglycosides, florfenicol and nalidixic acid, not commonly used in animal health and production showed no resistance (Piras et al. 2011, Li et al. 2014).

Antimicrobial resistance to at least one antibiotic was identified in 40.0% and 33.0% of clinical and food isolates, respectively. In the Southern Brazil, 82.0% of *Salmonella* samples of fresh pork sausage were resistant to at least one antibiotic tested (Mürmann et al. 2009). In the US, 47.0% of *Salmonella* Heidelberg isolates from human patients were resistant to antimicrobials (Han et al. 2011). *Salmonella* multidrug resistance is a growing concern since it limits treatment options for invasive disease, and it could lead to the transfer of resistance to other pathogenic organisms. In this study, a relative high frequency of multidrug-resistant *Salmonella* (25.0% of clinical isolates and 11.0% of food isolates) represents an additional public health risk, and *Salmonella* Schwarzengrund, in special, seemed to be resistant to six or more antibiotics.

The current gold standard method used to evaluate relatedness among *Salmonella* strains is PFGE has been established as important for the discrimination, characterization, and investigation of food-borne outbreaks (CDC 2010). Thus, PFGE fingerprinting shows several foods isolates that clustered with clinical isolates, mainly *Salmonella* Enteritidis and *Salmonella* Infantis among cities more than 500 miles apart, with persistence and geographical transmission in the Bahia State. However, other studies have described the limited discriminatory power of PFGE for *Salmonella* Enteritidis (CDC 2010, Mezal et al. 2014). A combination of different typing methods may be better to achieve best resolution for the discrimination of highly clonal organisms.

The combined use of different patterns of resistance and genotypes to analyze pathogenic isolates from food has been a useful way to identify the origins of outbreaks, establish sources of contamination, determine the etiological agents responsible for outbreaks, understand the epidemiology of food-borne disease and facilitate further studies on the spread of antibiotic-resistant isolates. This study provides data that indicate a probable transmission of *Salmonella* from food sources to cause infections in humans, establishing a serious risk to public health.

In this study, the most prevalent serotype in food samples was *Salmonella* Infantis and clinical samples of *Salmonella* Enteritidis. The most frequently observed resistance profiles were the NLX (nalidixic acid) and multi-resistance AMP / CFL / CRO / CTX / TET / TMS / NLX * (ampicillin, cephalothin, ceftriaxone, cefotaxime, tetracycline and trimethoprim / sulfametazol, nalidixic acid), common to isolates from food and clinical samples. In addition, clonal groups, found among strains epidemiologically unrelated, suggested that some isolated persisted in the environment over the years in different cities of Bahia. Thus, our results indicate that the clonal groups were both causing diseases and food contamination, emphasizing the need to maintain a system of surveillance for foodborne disease.

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